ZINC-CHITOSAN COMPOSITE COATING ON 316LSS FOR BONE TISSUE ENGINEERING BY ELECTROCHEMICAL METHOD

A Thesis Submitted in Partial Fulfillment
of the Requirement for the Degree of

Bachelor of Technology
in
Biomedical Engineering
by

NIRLIPTA SOVAN MISHRA

111BM0533

Under the supervision of

Dr. Amit Biswas

Department of Biotechnology & Medical Engineering
National Institute of Technology
Rourkela, Odisha-769008

May 2015

Department of Biotechnology and Medical Engineering
National Institute of Technology, Rourkela,
Odisha 769008, India

CERTIFICATE

This is to certify that the report entitled **"ZINC-CHITOSAN COMPOSITE COATING ON 316LSS FOR BONE TISSUE ENGINEERING BY ELECTROCHEMICAL METHOD"** submitted by **Nirlipta Sovan Mishra (111BM0533)** towards the partial fulfillment of the requirement for the degree of Bachelor of Technology in Biomedical Engineering in Department of Biotechnology & Medical Engineering, NIT Rourkela is a record of bonafide work carried out by him under my guidance and supervision**.**

Rourkela
Date

Dr. AMIT BISWAS
Dept. of Biotechnology and Medical Engineering
National Institute of Technology, Rourkela

ACKNOWLEDGEMENT

I would like to express my deepest gratitude to my project guide, **Dr. Amit Biswas**, Assistant Professor in the Department of Biotechnology and Medical Engineering, NIT Rourkela for believing in me and giving me the opportunity to work under him and lending me support at every stage of this project work. I am highly obliged to him for providing me with all necessary administrative facilities during the project work. I place on record my sincere gratitude to **Prof. Krishna Pramanik**, Head of Department, Department of Biotechnology and Medical Engineering, NIT Rourkela for her constant encouragement.

I also accord my thanks to **Ms. Sahely Saha, Mr. Krishna Kumar, Ms. Alisha Prasad, Mr. Subhashish Satpathy, Mr. Amartya Amitav** for their precious supervision, incessant support, inspiration and constructive criticism throughout my project work. I would also like to thank all the faculty members of the Department of Biotechnology and Medical Engineering, NIT ROURKELA. I wish to extend my sincere thanks to all my lab mates and friends for their help and support.

Nirlipta Sovan Mishra

111BM0533

CONTENTS

ABBREVIATIONS

316LSS	= 316L Stainless Steel
EDS	= Energy dispersive X-ray spectroscopy
EPD	= Electrophoretic deposition
FESEM	= Field emission scanning electron microscope
g	= Gram
min	= Minute
Pt	= Platinum
V	= Volts
%wt	= weight percentage
XRD	= X-ray diffraction
Zn	= Zinc

LIST OF FIGURES

LISTS OF TABLES

ABSTRACT

316LSS and the alloys of 316LSS are generally used for the fabrication of osteo-synthetic and orthopedic implants. In this project chitosan was chosen as the polymer to be coated on the 316LSS sample as an effort to induce surface modification of the sample. Because of its biodegradable nature, unique and multidimensional structural properties, chitosan has been extensively investigated and explored in the field of regenerative medicine and bone tissue engineering. Chitosan also exhibits the properties of low immunogenicity that makes it suitable to get accommodated in the body fluids. The bone implants used to treat bone disorders or to carry out tissue repair are susceptible to infections caused by staphylococci, specifically Staphylococcus aureus. Hence, the development of better biological materials that provide antimicrobial activity in bone tissue engineering is required. In this project fabrication of Zinc-Chitosan coating on 316LSS by electrochemical method was done for bone tissue engineering. A layer of Zinc was electroplated first on 316LSS at different supply voltages and at different deposition times. Then the as prepared Zn coated samples were characterized for their surface morphology and the samples on which Zinc was deposited in a particulate form in selected area over the substrate surface in minute amounts were further chosen for Chitosan coating. Deposition of chitosan on Zn-coated 316LSS was carried out by EPD method. Thereafter, the as prepared implant surfaces were characterized for their surface morphology through FESEM, elemental composition through EDS and phase purity through XRD. The coated samples were further placed in a petri plate with agar medium and their surfaces were exposed to E.coli culture. The petri plate was then kept in an incubator for nearly 36 hours and the samples were then tested for their antibacterial properties.

Keywords: 316LSS, Zinc, Chitosan, EPD, FESEM, EDS, XRD, E.coli

CHAPTER 1

INTRODUCTION

1 INTRODUCTION

The reported cases of bone disorders have increased steeply worldwide. These bone disorders are most prevalent in populations of age groups, who suffer from obesity, hyperglycaemia and poor physical performance. In such a scenario, bone tissue engineering has been perceived as a potential and significant alternative to the conventional bone grafts. The engineered bone tissue has advantages of limitless supply and no incidence of disease transmission. Bone tissue engineering encapsulates the fields of material science, medicine, structural design analysis like the Finite Element Analysis and Biomechanics.

A wide variety of materials like ceramic, metallic, polymeric and composite biomaterials are used for medical purposes. Among these materials, metallic biomaterials find application where load bearing structures are required such as in orthopaedics and dentistry. Both simple and complex shapes of these metallic biomaterials are relatively easy to fabricate by the help of well-established fabrication techniques such as forging, casting and machining. The various metallic biomaterials that find application in orthopaedics and dentistry include stainless steel, titanium, tantalum and nickel titanium alloys. Medical grade stainless steel (316LSS) is widely used in joint replacement prostheses and fracture repair implants. It is a more economical and accessible alternative as compared to other metallic biomaterials. The 316LSS implant placed inside the body is highly susceptible to corrosion and hence it needs to undergo surface modification techniques. One of the most suitable surface modification technique that can impart desirable surface morphology and surface chemistry to the 316LSS prosthesis is bioactive and biocompatible coating. These coatings may be may be thermally or electrolytically grown oxides, polymeric or elastomeric andhydroxyapatite. There are various surface modification methods or coating techniques like the mechanical methods, chemical methods, sol gel, anodic oxidation, physical vapour deposition, chemical vapour deposition, electrophoretic deposition, electroplating and biochemical methods [1]. EPD has many

advantages over other coating techniques like uniformity in coating, controlled thickness of the coating, simple apparatus and low deposition time [2]. Materials chosen for EPD coating are mainly biodegradable and bioactive charged polymers. The charged polymer molecules are aptly driven by the application of an electric field and consequently these molecules are condensed onto the metallic substrate [3]. Chitosan, Alginate, Gelatin, Polylactic acid (PLA), Polyglycolic acid (PGA), Poly (e-caprolactone) (PCL) and copolymers of polyglycolide are among the most significant biodegradable and bioactive polymers employed for coatings on implant surface. Chitosan coated 316LSS implant shows high mechanical strength, high compression strength, appropriate modulus of elasticity, high biocompatibility. Further the incorporation of Zinc into this system induces anti-microbial properties to the system.

OVERVIEW OF THE THESIS

The whole thesis has been divided into 6 chapters. In chapter 2, a review of literature has been carried out and previous works on different biomaterials, polymeric coatings and coating techniques have been discussed. In chapter 3, objectives of the project work have been discussed. In chapter 4, materials and methods for the project have been discussed. Chapter 5 enumerates the results and discussions with reference to the characterization of the coated samples. An outline of conclusions and all the references used have been enumerated in the last section of this thesis.

CHAPTER 2

LITERATURE REVIEW

2 LITERATURE REVIEW

Table 1: Different classes of biomaterials and their uses [4].

Class of materials	**Applications**
Metals and alloys	
316L Stainless Steel	Joint replacements, bone fracture fixation, heart valves, and electrodes.
Titanium and its alloys	Joint replacements, dental bridges and dental implants, coronary stents.
Co-Cr alloys	Joint replacements, Bone fracture fixation
Gold	Dental fillings and crowns
Silver	Pacemaker wires, suture materials, dental amalgams
Platinum	Electrodes, neural stimulation devices
Ceramics	
Aluminium oxide	Hip implants, dental implants, cochlear replacement
Zirconia	Hip implants
Calcium phosphate	Bone graft substitutes, surface coatings on total joint replacements, cell scaffolds
Carbon	Heart valve coatings, orthopaedic implants
Glass	Bone graft substitutes, filler for dental materials
Polymers	
Nylon	Surgical sutures, Gastrointestinal segments, tracheal tubes
Silicon rubber	Finger joints, artificial skin, breast implants, intraocular lenses, catheters
Polyester	Resorbable sutures, fracture fixation, cell scaffolds, skin wound coverings, drug delivery devices.
Polyethylene	Hip and knee implants, artificial tendons and ligaments, synthetic vascular grafts

PMMA PVC	Bone cement, intraocular lenses Tubing facial prostheses
Natural Collagen and gelatin Cellulose Chitin Hyaluronic acid Alginate	 Cosmetic surgery, wound dressing, cell scaffolds Drug delivery Wound dressing, drug delivery, cell scaffold Post-operative adhesion prevention, ophthalmic and orthopaedic lubricant, drug delivery, cell scaffold Drug delivery, cell encapsulation

2.1 Metallic biomaterials

They exhibit good biocompatibility, high mechanical strength and good load bearing capacity, corrosion resistance. The ideal metallic biomaterials or implants show appropriate modulus of elasticity as the bone and exhibit imaging transparency. Currently, the metallic biomaterials like 316LSS, Co-Cr alloys, Ti and its alloys are widely being used to repair the bone disorders [5].

2.2 316L Stainless Steel

Implants and prosthetic devices are subjected to various stresses. These parts need to have sufficient mechanical strength to withstand the stresses. So the implants should exhibit good mechanical properties and load bearing capacities. These implants may also corrode inside the body when they come in contact with the body fluids, acids and enzymes. So they should have high corrosion resistance. They should have good fatigue life and appropriate Young's Modulus of elasticity. 316LSS exhibits all these properties and hence is a suitable biomaterial for load bearing applications [5].

Stainless steel contains 18 wt% Cr and 8 wt% Ni so that it becomes stronger and more corrosion resistant than the Steel. Addition of Molybdenum (Mo) to SS further improves its corrosion resistance and then it was called 316SS. If the carbon content in the 316SS is high, then the Cr present in 316SS will react with Carbon leading to the formation of a compound, Chromium Carbide. Then the iron surface in stainless steel will be exposed to oxygen and water and hence it will corrode. So the carbon content in stainless steel has been reduced from 0.08 wt% to 0.03 wt%. On account of low carbon content, 316SS was further named as 316LSS. The presence of Chromium in Stainless Steel develops a passive oxide layer on the surface of the 316LSS. This passive oxide layer acts as a protective film, which prevents the metal underneath from getting exposed to oxygen and moisture, thus increasing the corrosion resistance of the 316LSS [5].

Table 2: Properties of 316LSS

	Yield Strength (MPa)	Ultimate Tensile Strength (MPa)	Young's Modulus (GPa)	Maximum elongation (%)
316L Stainless Steel	190	490	193	40

2.3 Bacterial infections (Microbiologically influenced corrosion) of 316LSS

Implant surfaces could be toxic in vivo if they undergo corrosion. Our body contains a large number of microorganisms that may grow on the implant surfaces and produce various metabolic by-products that may deteriorate the metallic implant surface. This phenomenon is known as bio corrosion. It is also referred to as Micro-biologically influenced corrosion. (MIC) [6]. 316LSS has been affected by various types of corrosion, mainly the pitting corrosion that can be activated by the presence of microorganisms. These microorganisms have the ability to

modify or mould the environment of the 316LSS, thus creating crevices or cracks and zones of differential aeration with the help of metabolite formation [7].

2.4 Antibacterial metals development

Bacterial attachment, growth and proliferation on the material surfaces needs to be prevented and for that the antibacterial metals development becomes an area of concern. [8]

According to a study, nine pure metals such as Ti, Co, Ni, Cu, Zn, Ag, Zr, Mo, Sn and Pb were tested of their antimicrobial properties against the two toxic bacterial strains of Gram positive Staphylococcus aureus and Gram negative E.coli. The results showed that different metals exhibited different antibacterial properties. While the metals like Zn, Ag and Cu exhibited high antibacterial properties, metals like titanium and tin did not at all exhibit antibacterial properties [8].

2.5 Zinc as an antibacterial element

Metal ions like Zn can be incorporated onto the implant material surface either by doping, electroplating or electro deposition [9], [10]. It has been shown in earlier works that Zn can induce mineralization of osteo-blast through Zn trafficking. This process involves Zn transporters and Zn storage proteins [11]. While Zn induces osteoblast mineralization, it also inhibits osteoclast differentiation, thus promoting osteo-blast activity bone formation [12], [13]. In previous works, it has been demonstrated that when the Zn^{2+} ions substituted fluoridated HAp coatings on 316LSS were tested against the bacterial strains like Staphylococcus aureus they destroyed those strains effectively [14].

2.6 Polymers for coating the 316LSS

2.6.1 Brief review of works on some polymeric coatings

It has been shown that Alginate-HAp composite scaffolds promote osteosarcoma cell adhesion [15]. Potential bone tissue engineered alginate gel beads can be prepared by introducing the alginate gel beads into Calcium phosphate cement [16]. Gelatin is derived from collagen

through its partial hydrolysis. It is a mixture of peptides and proteins. Gelatin promotes thrombogenicity and cellular proliferation [17]. Gelatin has high biocompatibility and better swelling ratio. So gelatin has been extensively used in drug delivery systems. Biocomposite scaffolds fabricated from Gelatin and bioactive nanoparticles of glass have porous three dimensional microstructures as observed under SEM. These scaffolds have density, porosity and elastic modulus very close to or in the range of natural bone [18]. So, gelatin promotes cell aggregation. In another work, a bioactive composite scaffold consisting of bioactive-glass and gelatin has been introduced through the method of direct foaming. This composite scaffold stands better than the polymer based scaffold as it allows direct bone tissue regeneration. So gelatin can be used to fabricate such composite scaffolds that can promote direct bone tissue regeneration. These scaffolds are biocompatible and non-degradable. They facilitate osteogenic differentiation and deposition of extracellular matrix [19].

Table 3: Advantages and limitations of alginate coatings

Advantages	Limitations
Delivery of osteo-conductive factors and bone forming cells.	Insufficient load bearing capacity in the initial stages of fixation.
Bone and cartilage regeneration due to their ability to fill irregularly shaped defects and ease of chemical modification.	Inherently degradable in physiological conditions.
	Poor mechanical properties

2.6.2 Properties imparted by different polymeric or other coatings

- Polypyrole and Nb_2O_5 nanoparticles: Enhanced biocompatibility, enhanced corrosion resistance and mechanical strength.

- HAp nanoparticles: Improved corrosion resistance in the body, promotes implant fixation by chemical bonding, excellent load bearing capacities and enhanced bioactivity.
- Heparin: Cell adhesion,anti-coagulation and inhibition of intimal hyperplasia.
- Plasma polymerised Allylamine (PPAA): Improved cell adhesion and cohesion properties.
- Poly (PDMA: Corrosion inhibition and improved cell adhesion.
- 3-octyl-thiophene)-(P3OT) and Polystyrene (PS): Enhanced cell adhesion.

2.6.3 Chitosan

Chitosan exhibits biocompatibility, biodegradability and osteo-conduction properties. Chitosan coatings exhibit good biocompatibility, low degradation and processing ease and they also have the potential to swell and dehydrate depending on composition and environment. They promote cell growth and have good mechanical strength, high compression strength (7.68MPa) and elastic modulus of 0.46 GPa well matched with the bones. They facilitate cell spreading and proliferation. It has been shown that the development of a biodegradable porous scaffold made from chitosan and alginate polymers enhances the mechanical and biological properties of the coating, facilitates the attachment of the bone forming osteoblasts readily to the chitosan-alginate coating, well proliferation of osteoblasts and high degree of tissue compatibility [21]. In previous works, it has been shown that chitosan and chitosan mediated coatings facilitate the immobilization of proteins, nucleic acids and virus particles and it has also been proved that the surface properties of the implants, precisely metallic implants have been positively affected by the chitosan coating [22]. Development of a chitosan-HA coating on 316LSS by EPD increases the corrosion resistance of the 316LSS sample [23]. In a previous work, Chitosan-bioactive glass composite layer was deposited on Titanium implants by EPD and the coated surface exhibited greater particle size, increased porosity and better corrosion resistance

[24]. It has also been shown that deposition of a chitosan-titania nanoparticle composite coating on 316LSS through EPD increases the corrosion resistance and hydrophilic tendency of the implant [25].

Table 4: Different polymeric coatings and the coating techniques for 316LSS substrate

Metal Substrate	Polymers or other coating materials used	Coating technique employed
Stainless steel	Polypyrole (PPy) and Nb_2O_5 nanoparticles	Electrophoretic deposition
	Chitosan	Spin coating, EPD
	HAp nanoparticles	Spin coating
	Heparin	Dip coating
	PlasmapolymerisedAllylamine	Low pressure plasma reactor
	PDMA/Fluoropolymers heterogenous blended coatings	Casting method
	Poly(3-octyl-thiophene)-(P3OT) & Polystyrene (PS)	Drop casting method

2.7 Electrophoretic deposition

It is the method by which the charged ions in the solution are attracted and coated onto the oppositely charged electrode under the influence of an electric field. It is a dual step process. Firstly, the charged ions move to the oppositely charged electrode under the influence of an electric field. Secondly, the particles that are stored on the electrode shape into a thick, compact film or layer that is further coated onto the metallic substrate.

Figure 1: Electrophoretic deposition setup

CHAPTER 3

OBJECTIVE

OBJECTIVE

The present work aims at the fabrication of a Zinc doped Chitosan coating on 316LSS for bone tissue engineering by EPD method. During the course of the work, the following objectives are to be fulfilled.

1. To develop a Zn coating on 316LSS sample and a Zn-Chitosan coating on 316LSS by EPD method and compare the two coatings.

2. To find out the optimal operating parameters (supply voltage and deposition time) for the deposition of Zn on 316LSS sample.

3. To study the effect of deposition time and supply voltage on the morphology of the coating surfaces.

4. To characterise the coated samples for their surface morphology analysis through optical microscopy and FESEM and phase purity analysis through XRD and to confirm the presence of Zinc on the coating by performing EDS analysis.

5. To check the antibacterial property induced by the Zinc and Chitosan coating on the 316LSS.

CHAPTER 4

MATERIALS AND METHODS

4 MATERIALS AND METHOD

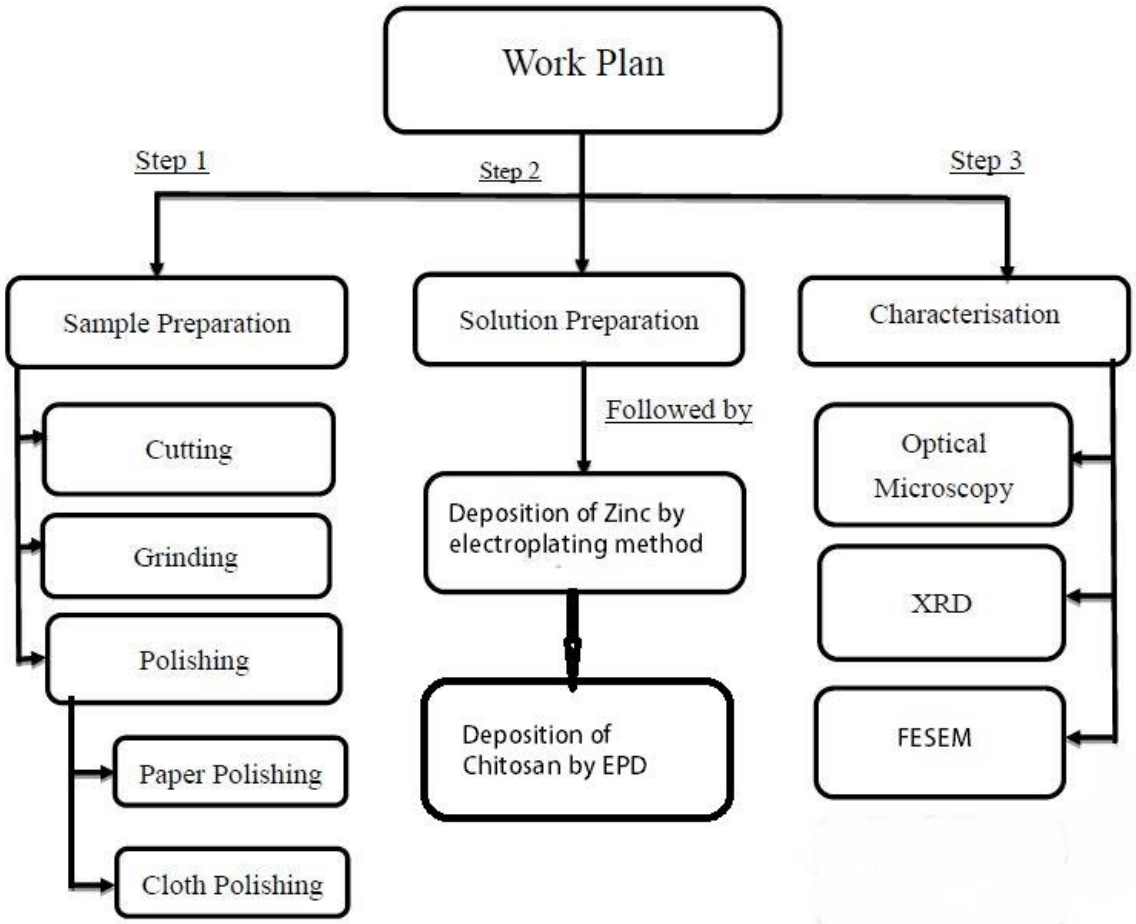

Figure 2: Schematic representation of the work plan

Materials and Chemicals required:

1) For sample preparation

   i) 316LSS (Stainless Steel sheet)

   ii) Belt grinder

   iii) SiC grit papers(1/0, 2/0, 3/0, 4/0)

   iv) Alumina paste

   v) Ethanol and distilled water for sample cleaning.

2) For electrochemical method to coat Zinc and Chitosan

i) A Nippo cell (contains pure Zinc electrode and Graphite electrode)

ii) Screw driver, hammer

iii) 250g/L $ZnSO_4$

iv) 0.4g Chitosan

v) Acetic acid (C_2H_5OH) and distilled water

vi) Magnetic stirrer

vii) Magnetic beads

viii) pH meter

ix) Regulated DC supply

3) For characterisation of the sample

i) Optical microscope

ii) XRD

iii) FESEM

4.1 Sample preparation

a) Cutting: A rectangular 316LSS sheet was cut into small square shapes of dimensions 1.5 cm x 1.5 cm. These square shaped 316LSS samples were considered to be the surface where coating is to be performed.

b) Grinding: These samples were then subjected to grinding in order to remove the native oxide layers, hydrocarbons in the air and other impurities present on their surfaces.

c) Grit-paper polishing: Various grades of SiC abrasive grit papers were used to perform the paper polishing of the samples. The paper polishing was done in four grades of SiC grit papers (1/0, 2/0, 3/0 and 4/0). In each and every grade of grit paper, longitudinal and transversal polishing was performed alternately till the scratches get aligned in one desired direction. This

paper polishing was continued until a mirror-polished surface was obtained so that a desirable surface for coating was achieved.

d) Cloth polishing: The samples were then subjected to cloth polishing using alumina powder (Al_2O_3) and water on the rotating disk.

e) Preventing abrasive pollution: The smoothing process may involve embedding of some abrasive particles into the metallic matrix of the samples of 316LSS. This phenomenon is generally referred to as abrasive pollution, which needs to be avoided. To achieve this objective samples were first washed with soap water followed by their cleaning in ultrasonic bath.

f) Rinsing with ethanol: The samples were dried after rinsing with ethanol.

g) The fully polished samples were then wrapped in tissue paper and placed inside a air tight box so as to avoid their oxidisation with air.

4.2 Coating of Zinc on the sample:

a) Procurement of Zinc electrode: A small Nippo cell was taken. Its outer metallic coating was removed with the help of a screw driver and hammer. After the outer coating was removed, a polymeric coating was encountered and subsequently removed. Then a cylindrical zinc electrode was extracted out of the system. This cylindrical Zinc electrode was purified by removing the graphite electrode and black coloured powder, MnO_2. Now this hollow cylindrical Zn electrode was purified using Ethanol.

b) Preparation of the electrolyte medium: 250g/L $ZnSO_4$ electrolyte solution was prepared by dissolving 12.5g of $ZnSO_4$ in 37.5ml of distilled water. The complete homogenous dissolution was obtained by stirring the solution in a magnetic stirrer using a magnetic bead. The operational parameters of the magnetic stirrer were kept at a rotating speed of 340 rpm and a temperature of 70 degree Celsius.

c) Electroplating of Zinc on the 316LSS sample: The pure Zinc hollow cylindrical electrode was used as anode while the steel sample was used as cathode. Then both these

electrodes were dipped in the $ZnSO_4$ solution in a beaker and provided with a regulated DC supply. The Zn deposition on 316LSS sample was carried out for the following process parameters:

Table – 5 – The process parameters for Zn deposition on 316LSS

Supply Voltage	Deposition time	
3V	10 min (Sample 1)	20 min (Sample 2)
4V	10 min (Sample 3)	20 min (Sample 4)
5V	10 min (Sample 5)	20 min (Sample 6)

Precautions to be taken while depositing the Zinc coating on 316LSS sample:

a) The red clamp (anode) and the black clamp (cathode) should not be allowed to touch each other.

b) Neither of the electrodes should be made to touch the walls of the solution beaker.

c) Equal area of the anode and cathode should be dipped in the solution.

d) Proper care should be taken that the clamps should not get dipped in the solution, or else iron may get leached out from the clamp and it may get deposited on the sample, thus forming an undesirable coating.

4.3 Deposition of Chitosan on Zinc-coated 316LSS by EPD:

a) Preparation of the Chitosan solution: 0.4g Chitosan was dissolved in the solution of 500ml acetic acid + water. To dissolve Chitosan in the solution, the solution is subjected to magnetic stirrer using a magnetic bead. The rotational speed of stirring was kept at 220 rpm and temperature of the stirring was maintained at 60 degree Celsius. The pH of this Chitosan solution was maintained between 3.3 – 3.6.

b) 150ml of this solution was taken in a beaker and EPD was then carried out.

c) The electrodes were washed with distilled water and then dried at room temperature.

d) Graphite rod was used as the anode and the Zn coated 316LSS sample was used as cathode. The Zn coated samples that exhibited globular deposition of Zn on 316LSS under the Optical microscopy were coated with Chitosan.

e) The Chitosan was deposited at a supply voltage of 25 V for deposition time of 10 min and 20 min.

Table – 6 - The solution composition and EPD process parameters

Electrolyte	Acetic acid – 5ml Water – 495ml
Polymer used	Chitosan – 0.4g
pH	3.3 – 3.6
Applied voltage	25 V
Deposition time	10 min, 20 min

Table – 7 - Process parameters for EPD of Chitosan on Zn coated 316LSS sample

Optimal Zn coated sample	Operational supply voltage	Chitosan deposition time	
To be identified through optical microscopy	25V	10 min	20 min
		10 min	20 min

4.4 Characterisation techniques

Characterisation technique like surface morphology analysis was carried out by optical microscope. An FESEM was also used to analyse the surface morphology. Phase purity analysis was performed using an XRD. The elemental compositions of the coatings were determined using EDS.

4.4.1 Surface morphology analysis (Optical Microscope):

An optical microscope - ZEISS, Axiotech with an image analyser was used for surface morphology analysis of the Zn-coated 316LSS sample. The software used for the surface

morphology analysis was Axovision 4.8. The prepared sample was placed over the horizontal stage with surface perpendicular to the optical axis of the optical microscope. Light from a source was used to illuminate the sample through the objective lens. This light was then focussed by a condenser lens into a beam, whose orientation was adjusted parallel to the optical axis of the optical microscope with the help of a half silvered mirror. The path of the light can be described as under:

Source → Zn coated 316LSS sample through objective lens → Reflection from the sample surface → Objective → Half silvered mirror → Eyepiece → Camera port.

Figure 3: Optical Microscope set up

4.4.2 Phase purity analysis

The phase identification and crystallinity of the coated material was analysed by the XRD that is a technique used to characterise the compounds based on the diffraction pattern of the constituent elements. The X-ray diffractometer, X'PERT PANalytical XRD and the X'PERT data collector software were used to perform the XRD characterisation of the sample. CuK-radiation was performed by utilising a Cu target as X-ray source. The background noise in the detector was eliminated by spotting a graphite monochromator before the relative counter. The XRD profile was obtained in the range of 10 and the scan rate was maintained at

3^0/min. The XRD profiles of the Zn coated 316LSS sample and Zn–Chitosan coated 316LSS sample thus obtained were compared with the standard JCPDS files of Zn, Chitosan and austenitic stainless steel. The peaks obtained in the diffraction pattern and the corresponding angles were studied and compared with the standard JCPDS so as to identify the phases present in the coatings.

Figure 4: X'PERT PANalytical X-ray diffractometer

4.4.3 FESEM

The Zn coated 316LSS sample – Sample 1 and Sample 3 and a Zn – Chitosan coated 316LSS sample were characterised using FESEM technique. The surface needed to be made conductive for performing FESEM. While Zn coated 316LSS surface was a conductive surface, deposition of a layer of Chitosan on the Zn coated 316LSS sample made its surface nonconductive. To make the surface conductive, Pt sputtering was performed on the non-conductive Chitosan coated 316LSS surface. By FESEM, information about the surface morphology of the coated

sample was obtained. Alongside FESEM, the EDS was also performed. With the help of EDS, the information about the elemental composition of the coated surface was obtained.

4.4.4 Antibacterial effect testing

- Agar medium was prepared for culturing E-Coli.
- Media preparation: In 100 ml of water, 2.5 g of Luria Bertani Broth was added, then 3 g of agar was added to the above solution. The solution was mixed thoroughly and was autoclaved at 121^{o} C for 15 minutes.
- Under Laminar air flow hood, 20 ml of autoclaved media was poured into three petriplates each. Media was poured carefully before it solidified. The petriplates were kept in rest for 10-15 minutes until agar was solidified.
- Using 1ml tip of micropipette, 3 equidistant wells were cut in the solidified media. 1 well was left for positive control, a polished 316LSS sample.
- In the other two wells, Zn-coated 316LSS sample and Zn-Chitosan coated 316LSS sample were kept.
- 1ml of E-Coli culture was added in the centre of the petriplates and spread evenly with an autoclaved L-shaped rod. The petriplates were wrapped tightly with parafilm and labelled respectively.
- The inoculated petri plates were kept in incubator at 35^{0}C overnight. In the following day petri dishes were inspected for Zone off Inhibition.

.

CHAPTER 5

RESULTS & DISCUSSION

5. RESULTS AND DISCUSSION

This work was aimed to have an investigation of the deposition of Zn and Chitosan on 316LSS by electrochemical method. The coated surfaces were then characterised to check any morphological alteration, to identify different phases, to check the elemental composition and to confirm the presence of desirable coating on the 316LSS sample. The results obtained from different characterisation techniques are discussed in details in the following section.

5.1 Optical microscopy

Figure 5(a), 5(b), 5(c) and 5(d) below show the optical microscopy images of Zn coated 316LSS samples at two different supply voltages of 3V and 4V and at two different deposition times of 10 min and 20 min. Out of these samples, images of sample 1 and sample 3 showed that in those samples Zn was distributed in minute amounts over the 316LSS surface resulting in a non-uniform coating so that the whole surface area of the 316LSS did not get covered by Zn coating and certain surface area of the substrate surface was left for Chitosan deposition. Since minute amount of Zn deposition on 316LSS surface was desirable only to induce anti-bacterial effects, sample 1 and sample 3 were chosen for further Chitosan deposition. Here the Zinc was present on the surface of 316LSS in dispersed phase. So sample 1 and sample 3 were further chosen as optimal samples for carrying out the chitosan coating by EPD. Figure 5(e) and 5(f) show the optical microscopy images for the Zn-Chitosan coated 316LSS samples. Moreover, deposition time in EPD process has a strong effect on the morphology of the coated layer. It has been observed that the deposition time affects the thickness of the coating layer. It has been clearly depicted that while depositing Zinc on 316LSS, with increase in deposition time at a constant supply voltage, deposition increases linearly and with an increase in supply voltage at a constant deposition time also, the deposition increases. Then in both the cases, the deposition rate further decreases with the increase in one parameter (keeping the other constant) and finally saturates at very high temperature.

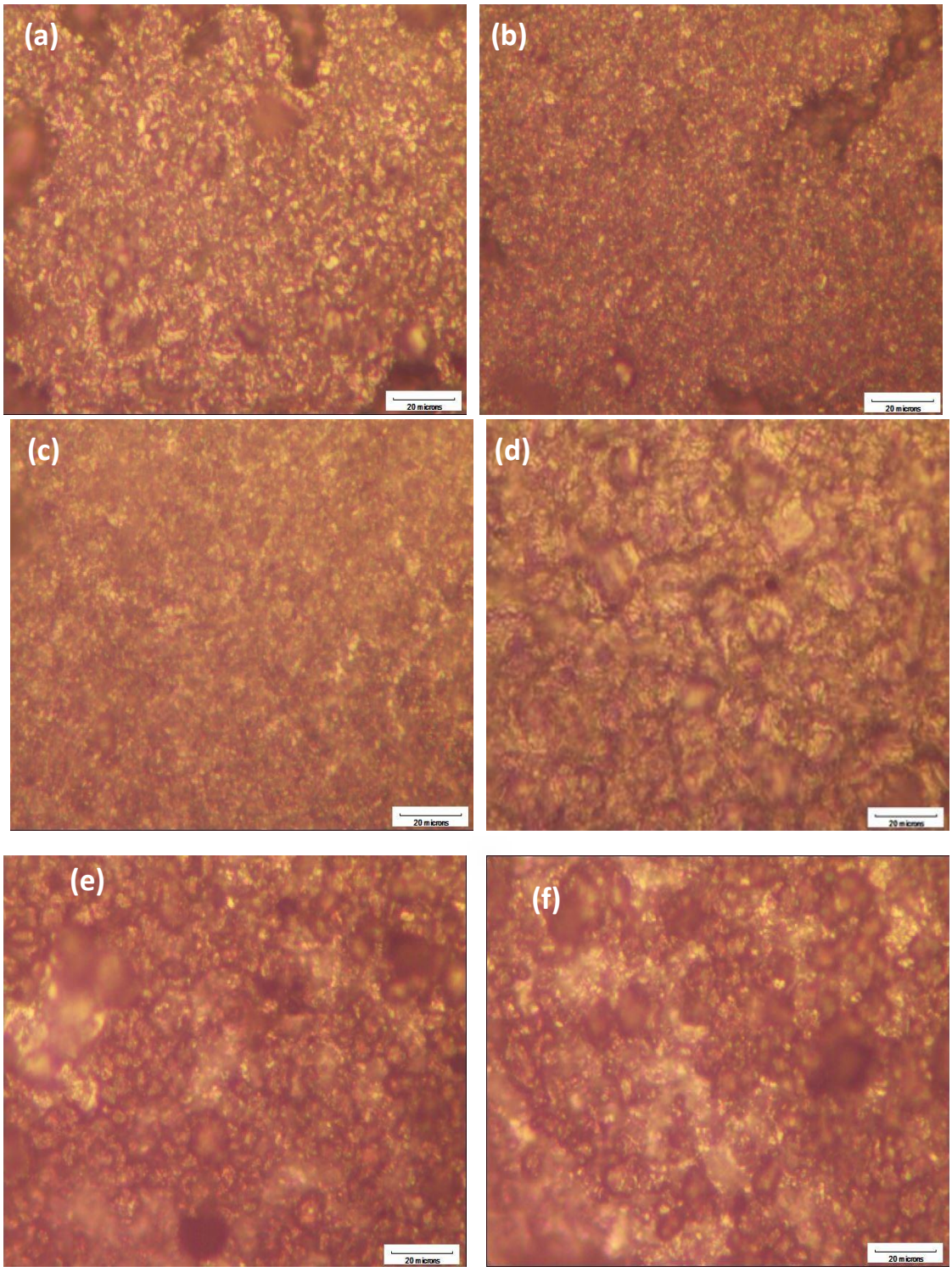

Figure 5: Optical microscopy image for Zn coated Sample (a) 3V- 10 min, (b) 3V-20 min, (c) 4V, 10 min, (d) 4V-20 min (e) Zn - Chitosan coated sample (Zn at 3V, 10 min Chitosan at 25V, 10 min) & (f) Zn - Chitosan coated sample (Zn at 4V, 10 min Chitosan at 25V, 10 min)

5.2 XRD Analysis

The XRD profile of Zn coated stainless steel sample with deposition supply voltage of 4V and deposition time of 10 min was generated. Further this coated sample on which deposition of chitosan was carried out at process parameters like supply voltage of 25V and deposition time of 10 min was characterised by XRD and its XRD profile was generated.

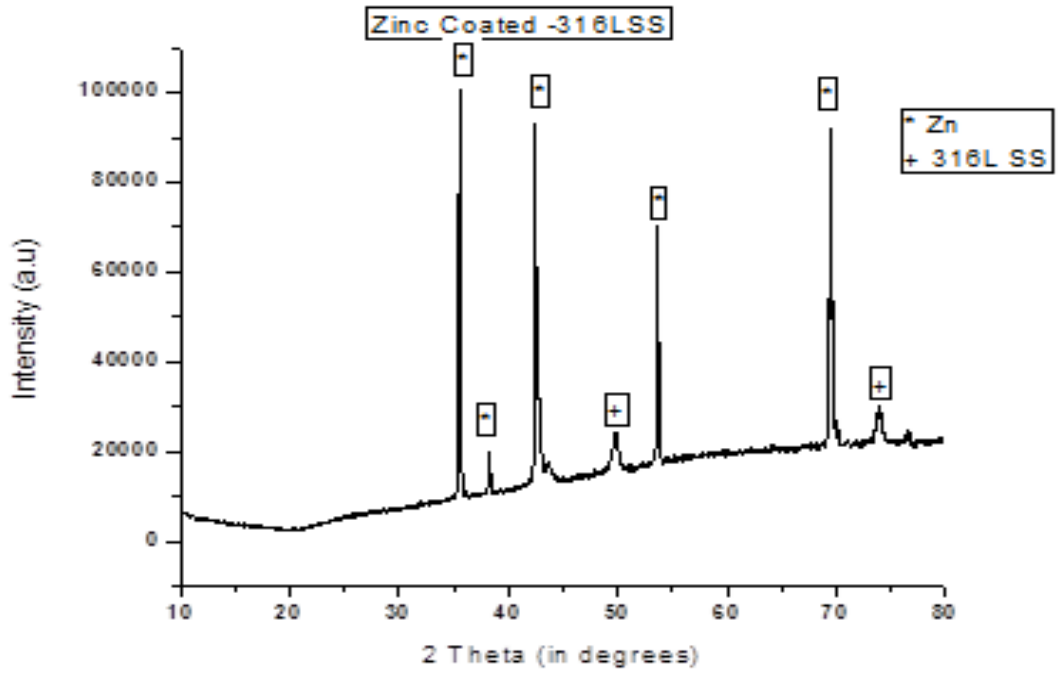

Figure 6(a): XRD profile of Zn coated 316LSS

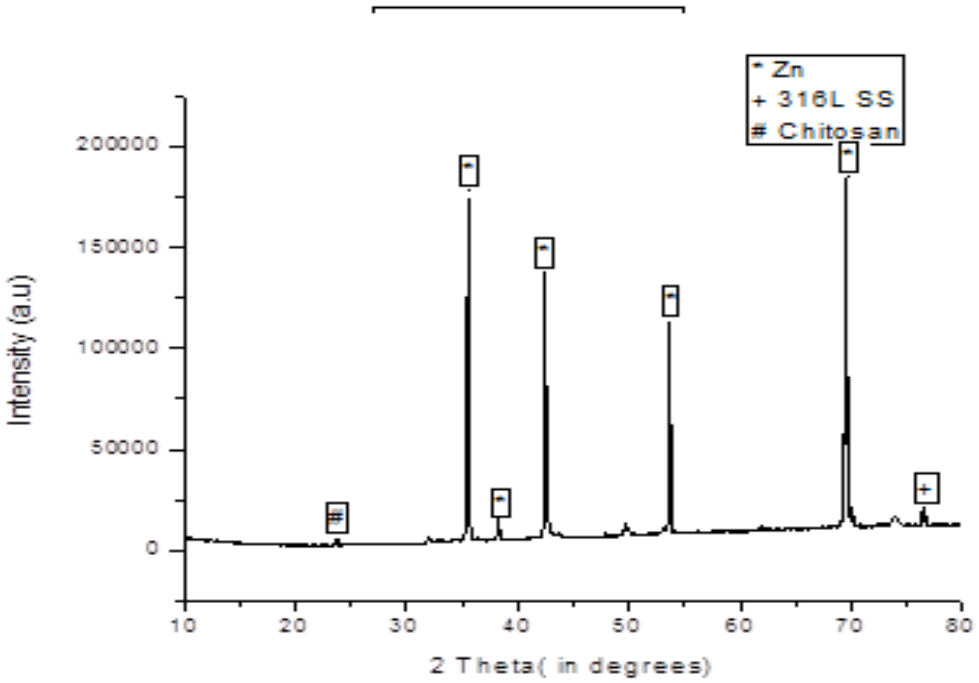

Figure 6(b): XRD profile of Zn-Chitosan coated 316LSS

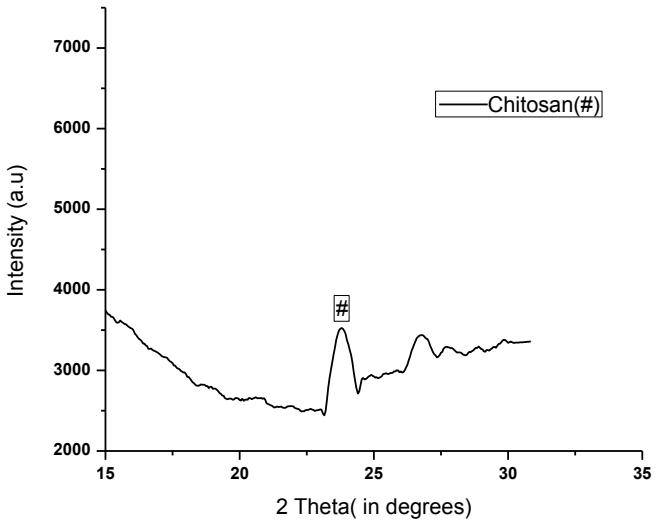

Figure 6(c): XRD profile of Zn-Chitosan coated 316LSS in the range of $2\theta=15^0$ to $2\theta=30^0$ to show the peak of Chitosan

Figure 6(a) shows the XRD profile of Zn coated 316LSS. Here high intensity peaks were observed at 2θ values of 35.69^0, 42.52^0, 53.64^0 and 69.66^0. According to the JCPDS of Zn, the XRD peaks of Zn are obtained at 2θ values of 38^0, 42^0, 54^0 and 69^0. Thus it is clearly depicted that Zn has been successfully coated on the surface of 316LSS sample. Comparatively lesser intensity peaks of austenitic stainless steel were observed at 2θ values of 76.63^0 and 49.77^0. These peaks correspond to 316LSS. According to the literature, in stainless steel without coating, a very high intensity peak is observed at $2\theta = 73^0$ in the XRD pattern. In our XRD data for Zn coated 316LSS, a low intensity peak was observed at 73.95^0. Here peak broadening occurred due to the Zn coating on 316LSS. In the XRD profile of 316LSS, a peak broadening at $2\theta = 49.64^0$ was observed. This might have occurred due to the overlapping of planes or shifting of bonds.

Figure 6(b) shows the XRD profile of Zn-Chitosan coated 316LSS sample surface. Here high intensity peaks were observed at $2\theta = 42.66^0$, 35.84^0, 53.78^0, and 69.66^0. All these diffraction angles correspond to the peaks observed in the JCPDS of Zn. A peak was also observed at $2\theta = 23.7^0$, which corresponds to the JCPDS of Chitosan that shows a standard XRD peak of Chitosan at $2\theta = 22.50$. In our XRD profile, a low intensity peak was also observed at $2\theta = 38.37^0$. Very low intensity peaks were $2\theta = 49.49^0$, 73.82^0 and 76.63^0. According to the standard JCPDS of non-coated polished stainless steel, medium and low intensity peaks are observed at $2\theta = 52^0$ and 77^0 respectively. So peaks at $2\theta = 49.49^0$ and 76.63^0 correspond to the austenitic stainless steel.

5.3 FESEM analysis for surface microstructure

From figure 5(a), 5(b), 5(c) and 5(d), it is quite clear that deposition time and supply voltages affect strongly the amount of coating material that gets deposited onto the surface. At 4V supply voltage and 10 min deposition time, non-uniform and minute deposition of Zn was observed. So, Chitosan deposition at 25V supply voltage and deposition time of 10 min was carried out

on this Zn coated sample by EPD method. This Zn-chitosan coated sample under FESEM exhibited fine coating structure confirming presence of both Zinc and Chitosan on the coating surface.

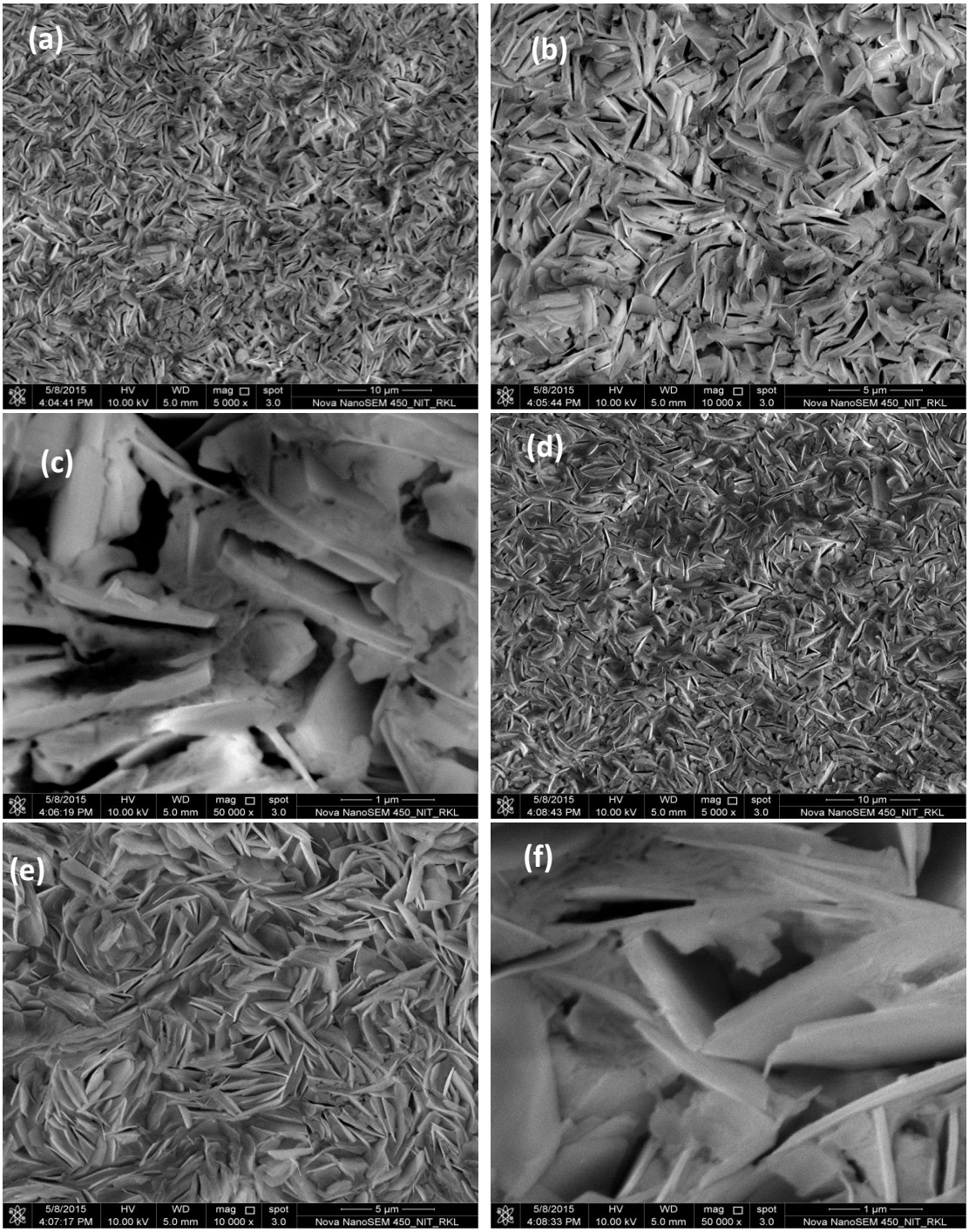

Figure 7: FESEM image for Zn coated 316LSS (a) at 3V, 10 min (5000x), (b) 3V, 10 min (10000x), (c) 3V, 10 min(50000x), (d) 4V, 10 min(5000x), (e) 4V, 10 min(10000x), (f) 4V, 10 min(50000x)

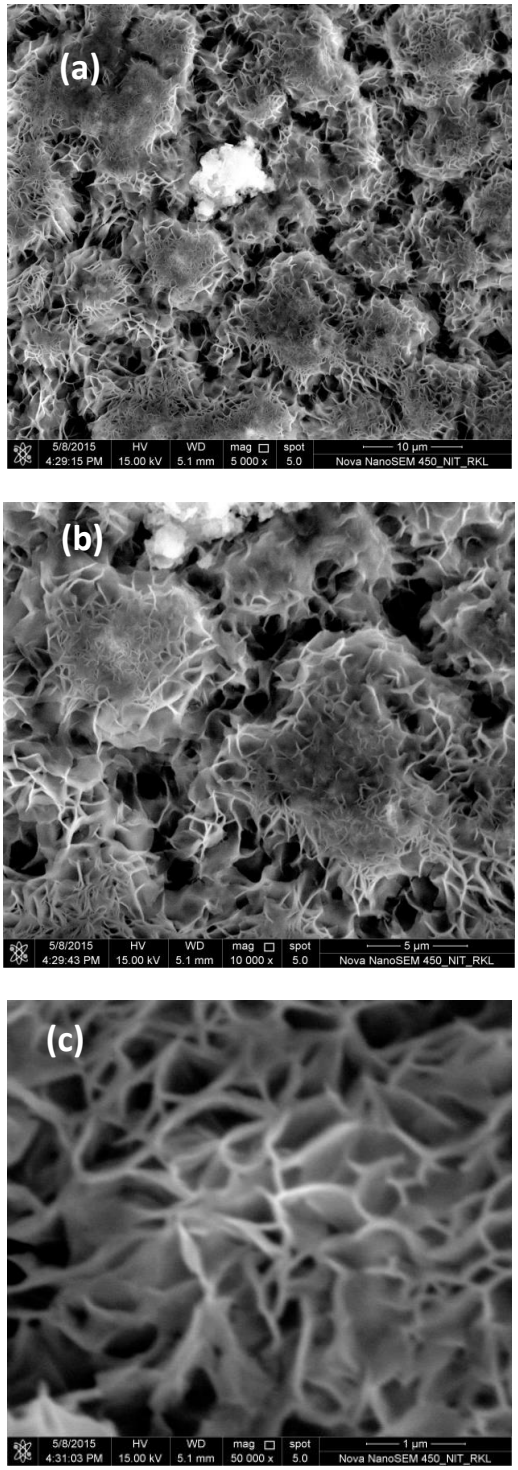

Figure 8: FESEM image for Zn-Chitosan coated sample, Zn – 4V, 10 min and chitosan – 25V, 10 min (a) 5000x, (b) 10000x and (c) 50000x

5.4 EDS analysis

Table 8: EDS data analysis of Zn coated sample

El	AN	Series	Net unn. C [wt.%]	norm. C [wt.%]	Atom. C [at.%]	Error (1 Sigma) [wt.%]
Zn	30	K-series	14117	61.49	71.76	40.66 2.14
O	8	K-series	13549	15.07	17.58	40.72 2.05
S	16	K-series	12996	5.21	6.08	7.02 0.22
C	6	K-series	807	3.03	3.53	10.89 0.75
Fe	26	K-series	647	0.90	1.05	0.70 0.07
Total:			85.69	100.00	100.00	

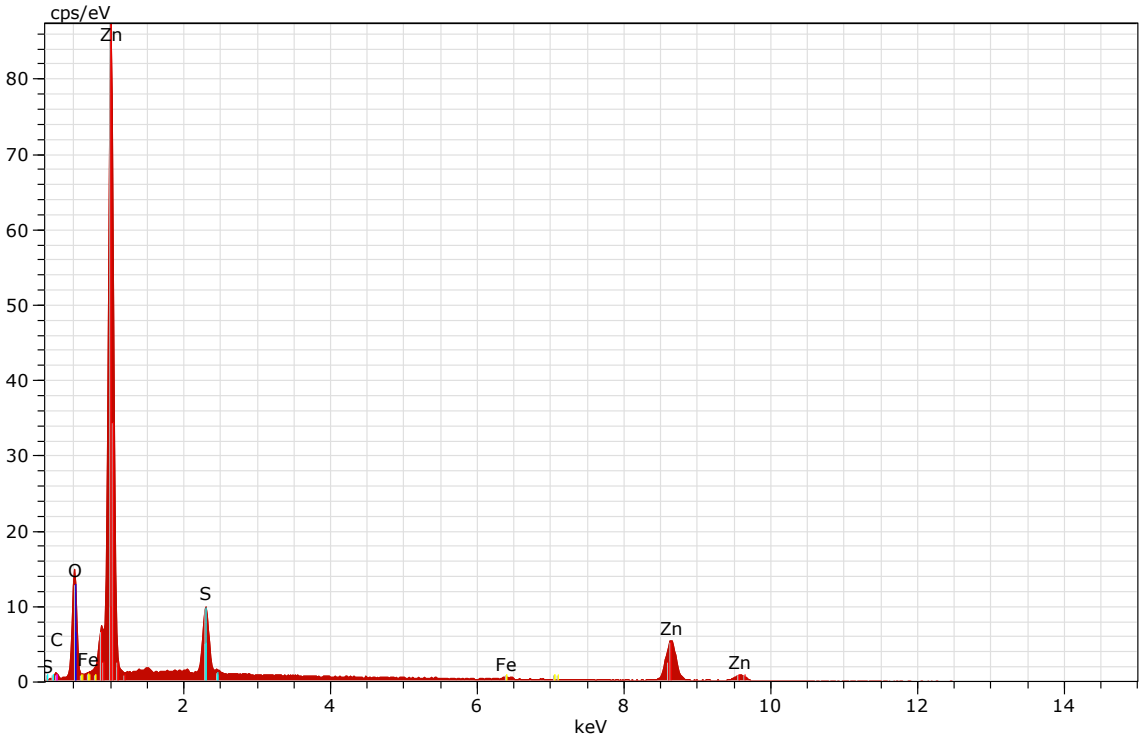

Figure 9: EDS plot for Zn coated 316LSS sample

Discussion: The EDS analysis of the Zn coated sample confirmed the presence of high amounts of Zn on the coating surface. Zn peak of around 90 cps/keV was observed at an energy of

1 keV. Small Zn peaks were also observed at 8.6 keV and 9.7keV. It also indicated small peaks of Iron (Fe), Carbon(C), Oxygen (O) and Sulphur (S).

Table 9: EDS data analysis of Zn-Chitosan coated sample

El	AN	Series	Net unn. C [wt.%]	norm.C [wt.%]	Atom. C [at.%]	Error (1 Sigma) [wt.%]
Zn	30	K-series	20880	75.05	89.76	66.04	2.57
O	8	K-series	6158	5.90	7.06	21.22	0.92
C	6	K-series	850	2.66	3.18	12.74	0.66
Pt	78	M-series	3994	0.00	0.00	0.00	0.00
Total:			83.61	100.00	100.00		

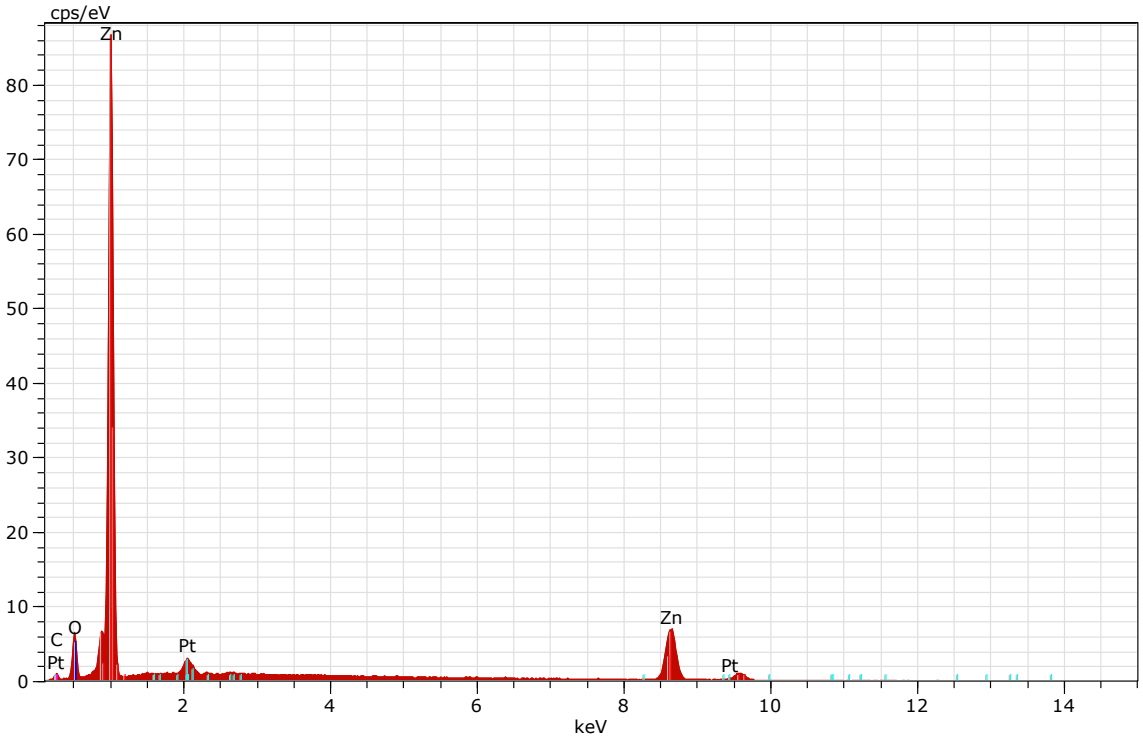

Figure 10: EDS plot for Zn - Chitosan coated 316LSS sample

Discussion: The EDS analysis of the Zn-Chitosan coated sample exhibited Zn peaks at 1keV and 8.6 keV confirming the presence of Zn in the coating. It also exhibited peaks of Carbon and Oxygen. It also confirmed that the maximum surface area of the 316LSS had been coated with Zn and Chitosan as no peak of Fe was observed. Very small cps peaks of Pt were also observed. These peaks were present because of the platinum coating process that was carried out on the Zn-chitosan coated 316LSS sample to make it a conductive surface for characterisation through FESEM.

5.5 Antibacterial effect testing results after 36 hours of incubation

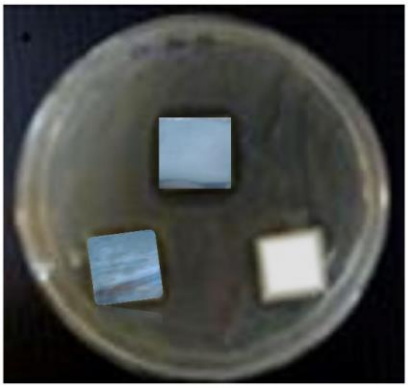

Figure 11: Image of the samples in the petri plate after 36 hours of incubation

The sample at the top is the Zinc coated sample, the sample at the bottom left is the Zinc-Chitosan coated sample and the sample at the bottom right is the control sample (polished 316LSS). It is clearly observable that the polished 316LSS sample does not significantly clear out the bacterial zone, whereas a significant portion of the bacterial culture in the petri plate has been cleared out by both the Zn-coated 316LSS sample and the Zn-Chitosan coated 316LSS sample. This confirms the antibacterial property of the Zinc and hence Zinc can be used in association with a natural biopolymer like chitosan to induce antibacterial effects in the sample.

CONCLUSIONS

CONCLUSIONS

After carrying out all the processes as per the work plan and analysing all the results and observations, a conclusion can be drawn that the Zn and Chitosan layers were successfully deposited on 316LSS by electrochemical method.

- The implant surface property required for better bioactivity was increased as inferred from the result of optical microscopy. An apt Zn-Chitosan coating was obtained at Zn deposition parameters of 4V, 10 min and on this sample Chitosan was deposited at supply voltage of 25V and deposition time of 10 min.
- The deposition time and supply voltages have an effect on the amount of coating material deposited as studied through the optical microscopy and FESEM.
- The presence of Zinc and Chitosan on the coatings of the specimen samples was confirmed by the XRD analysis. The presence of Zn in the coating was also confirmed by the EDS analysis.
- The antibacterial property of the Zinc was confirmed by checking the zonal clearance of a region of bacteria in the petri dish near the vicinity of the Zinc coated 316LSS.

REFERENCES

REFERENCES

1. S.H. Lee, H.W. Kim, E.J. Lee, *Hydroxyapatite-TiO_2 hybrid coating on plasma nitride 316L stainless steel*, Vacuum, 86, (2012), 1402-1407
2. Kapil D. Patel, Rajendra K. Singh, Eun-Jung Lee, Cheol Min-Han, *Tailoring solubility and drug release from electrophoretic deposited chitosan-gelatin films on titanium*, Surface and coatings technology, 242, (2013), 232-236
3. T. Yoshioka, A. Chavez-Valdez and J.A Roether, *AC electrophoretic deposition of organic-inorganic composite coatings*," Journal of colloid and interface science, 392, (2013) 167-171, .
4. Ajay Saxena and Abhinandan Rout, *Study of HAp and HAp-Chitosan composite on 316LSS by electrophoretic deposition*, ethesis, NIT Rourkela, (2011)
5. Li-Ping He and Hairong Liu. *Fabrication of HAp/Ni biomedical coatings using an electro-codeposition technique*, Surface and Coatings Technology, 160 (2002) 109-113
6. Kurissery R. Sreekumari, Yoshihiro Sato and Yasushi Kikuchi, *Antibacterial metals – a viable solution for bacterial attachment and microbiologically influenced corrosion*, Materials Transactions, 46 No. 07 (2005) 1636-1645
7. Ibars J R, Moreno DA, Ranninger C., "Microbial corrosion of Stainless Steel, Microbiologia, 8(2), (1992), 63-75
8. Yasuyuki M, Kinihiro K, Kurissery S, Kanavilly Y N, Sato Y, *Antibacterial properties of nine pure metals: a laboratory study using Staphylococcus aureus and E.coli*, Biofouling, 26(7), (2010), 851-858
9. K Sahithi, M.Swetha, M. Prabaharan and others, *Biocomposites containing natural polymers and hydroxyapatite for bone tissue engineering*, International Journal of Biological macromolecules, 47(1), (2010), 1-4

10. S.Saravanan, S.Nethala, S.Pattnaik, A.Tripathi, A.Moorthi, *Preparation, characterization and antimicrobial activity of a biocomposite scaffold containing chitosan/nano-hydroxyapatite/nano-silver for bone tissue engineering*, International Journal of Biological Macromolecules, 49 (2), (2011), 188-193

11. RameshNiranjan, C. Koushik, *A novel injectable temperature sensitive zinc doped chitosan/beta – Glycerophosphate hydrogel for bone tissue engineering*, Int. J. Biol. Macromolecules, 54, (2012), 24-29

12. M.Yamaguchi, H.Oishi, Y. Suketa, *Zinc stimulation of bone synthesis protein in tissue culture*, Biochemical Pharmacology, 37, (1988), 4075-4080.

13. M. Andrews, C. Gallagher, Allred, *The role of Zinc in wound healing*, Advances in wound care, 12 (1999), 137-138.

14. J. Liu, H. Dong, J. Buhagiar, C.F. Song, B.J. Yu, L.M. Qian, Z.R. Zhou, Effect of low temperature plasma carbonitriding on the fretting behaviour of 316LVM medical grade austenitic stainless steels, *wear2010.12.017*, (2010)

15. Krajewska, Barbara, Application of chitin-and chitosan-based materials for enzyme immobilizations: a review, *Enzyme and microbial technology* 35, no. 2 (2004): 126-139.

16. Yusof, Nealda Leila Binte Muhammad, Aileen Wee, Lee Yong Lim, and Eugene Khor, *Flexible chitin films as potential wound-dressing materials: Wound model studies*, Journal of Biomedical Materials Research *Part A* 66, no. 2 (2003): 224-232.

17. Tomihata, Kenji, and Yoshito Ikada, *In vitro and in vivo degradation of films of chitin and its deacetylated derivatives*, Biomaterials 18, no. 7 (1997): 567-575.

18. Kato, Yoshinori, Hiraku Onishi, and Yoshiharu Machida, *Application of chitin and chitosan derivatives in the pharmaceutical field*, Current Pharmaceutical Biotechnology 4, no. 5 (2003): 303-309.

19. Levengood, Sheeny K. Lan, and Miqin Zhang, *Chitosan-based scaffolds for bone tissue engineering*, Journal of Materials Chemistry *B* 2, no. 21 (2014): 3161-3184.

20. Yang, Jun, Teng Long, Nan-Fei He, Ya-Ping Guo, Zhen-An Zhu, and Qin-Fei Ke, *Fabrication of a chitosan/bioglass three-dimensional porous scaffold for bone tissue engineering applications*, Journal of Materials Chemistry *B* 2, no. 38 (2014): 6611-6618.

21. Yang, Jun, Teng Long, Nan-Fei He, Ya-Ping Guo, Zhen-An Zhu, and Qin-Fei Ke, *Fabrication of a chitosan/bioglass three-dimensional porous scaffold for bone tissue engineering applications*, Journal of Materials Chemistry *B* 2, no. 38 (2014): 6611-6618.

22. F. Gebhardt, S. Seuss, M. C. Turhan, H. Hornberger, *Characterisation of electrophoretic chitosan coatings on stainless steel*, Materials letters, *66, (2012)302-304*

23. A.W.G. Nijhuis, S.C.G. Leeuwenbergh, J.A. Jansen, *Wet chemical deposition of functional coatings for bone implantology*, Macromolecular bioscience, *10, (2010), 1316-1329*

24. T.M. Sridhar, U.K, *Preparation and characterisation of electrophoretically deposited hydroxyapatite coatings on type 316L stainless steel*, Corrosion science, *45, (2003) 237-252*

25. A. Balmurugan, B.G., *Electrochemical and structural characterisation of zirconia reinforced hydroxyapatite bioceramic sol gel coatings on surgical grade 316L stainless steel for biomedical applications,"* Ceramics International, 33,(2007), 605-614